COSOLVENT CONTROL OF SUBSTRATE INHIBITION IN COSOLVENT STIMULATION OF β-GLUCURONIDASE ACTIVITY

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SUMMARY: Bacterial β-glucuronidase exhibits a considerable increase in specific activity, upon addition of suitable organic cosolvents to aqueous systems. The effect is especially marked with three to five carbon alcohols; a nine fold increase was observed in this study using t-butanol in the range of 10% by volume of t-butanol in water. Most such increases were previously ascribed to cosolvent effects on V_{max}, i.e. to the catalytic steps. However, it is shown here that this enzyme is susceptible to marked substrate inhibition, and that half of the cosolvent induced activity increase is due to decreasing this inhibition. The other 50% of the activity increase probably does reside in cosolvent stimulation of V_{max}.

Although most *in vitro* work with enzymes involves work with neat water as the principal or sole solvent, it is commonly agreed that many enzymes probably exist *in vivo* in an environment which is the equivalent of a solvent that may be rather less nonpolar than water alone, with its supporting electrolytes. For instance, membranes and cytosols might provide such a mileu; the equivalent of water with considerable organic material dissolved in it. The possible consequences of this to enzyme activity has been exemplified by β-glucuronidase. In 1958 Sigma Chemical Co. issued a bulletin (1) pointing out that small amounts of chloroform caused a considerable increase in the activity of *E. coli* β-glucuronidase. Gautney, Barker, and Hill (2) carried out an organized investigation of this system in 1959, utilizing several organic solvents. Later, Tan and Lovrien (3) extended this kind of work to six additional enzymes, using an assortment of alcohols, and dioxane rather than chloroform. However, Tan and Lovrien assigned most of the effect exerted by such cosolvents to the V_{max} term, without further analysis. The present work is a more detailed study of the *E. coli* β-glucuronidase enzyme, in the water-t-butanol solvent system. It now appears that although t-butanol's effect on V_{max} remains important, of about

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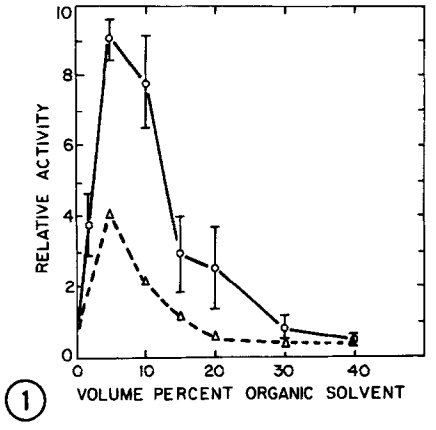

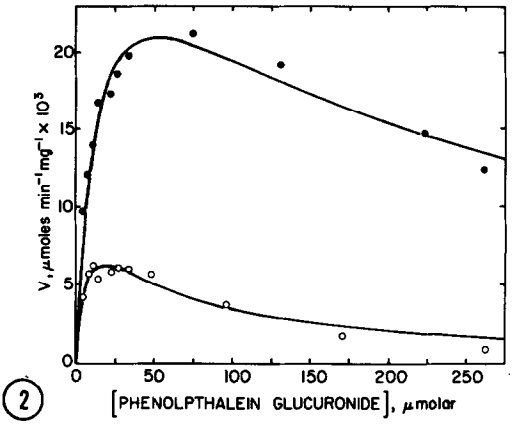

Fig. 1: Organic solvent dependent activity of E. coli β-glucuronidase relative to that in buffer only at 25° C. The concentrations in the assay prior to addition of glycine buffer were: Enzyme 0.2 mg/ml; phenolphthalein β-D-glucuronic acid; 330 μM; varying amounts of water and organic solvent so as to achieve the desired concentration of organic solvent; 0.28 M sodium acetate (pH 5.0). O: t-butanol Δ: p-dioxane

Fig. 2: Rate of hydrolysis of phenolphthalein β-D-glucuronic acid in the presence (Δ) and absence (o) of 5% (v/v) t-butanol at 25° C. The solid line is a nonlinear least squares fit to Eq. [2]. The concentrations in the assay prior to addition of glycine buffer were: Enzyme: 0.3 mg/ml; 0.3 M sodium acetate (pH 5.0), alcohol and substrate concentrations were as noted.

equal importance is its ability to lift substrate inhibition, to which the enzyme is susceptible.

MATERIALS AND METHODS

Phenolphalein β-D-glucuronide and E. coli β-glucuronidase type VI A were obtained from Sigma Chemical Co. t-Butanol was distilled twice and recrystallized. Peroxides were removed from p-Dioxane by refluxing over $LiAlH_4$ for 2 days followed by distillation.

β-Glucuronidase activity was measured by a modification of the method described by Fishman et al. (4). Phenolphthalein β-D-glucuronide was incubated with enzyme in 0.28 M sodium acetate buffer pH 5.0 at 25°C. At various times, 1 ml aliquots were withdrawn and added to 1.0 M glycine buffer pH 10.5. Phenolphthalein was then determined spectrophotometrically at 553 nm. Protein concentration was determined using Hartree's modification (5) of the Lowry method.

RESULTS AND DISCUSSION

The effect of t-butanol and p-dioxane concentration on β-glucuronidase activity is shown in Fig. 1. The rate of hydrolysis of phenolphthalein glucuronide rises to a maximum (amounting to a nine-fold activation for t-butanol),

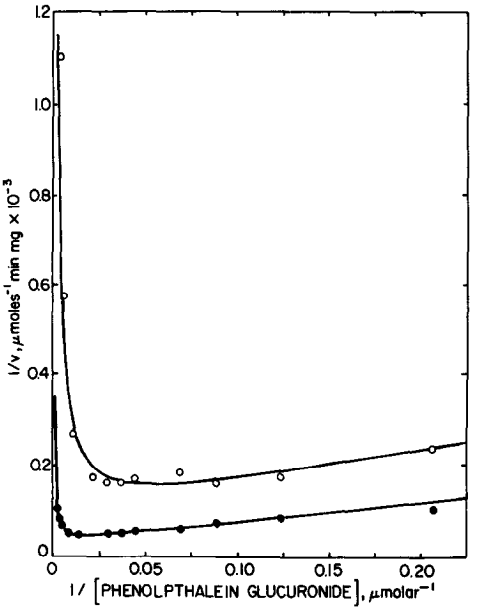

Fig. 3: Lineweaver-Burk plot of data from Fig. 2; reciprocal of Eq [2]. The solid line is a replot using parameters obtained from a nonlinear least squares fitted to Eq [2]. Conditions are the same as those for Fig. 2.

in the region of five volume percent organic solvent, then falls off at higher concentrations. This is consistent with the work of Gautney, Barker and Hill (2) who obtained an eight-fold activation with n-amyl alcohol.

Figure 2 plots the rates of hydrolysis of the substrate as a function of initial substrate concentration in the presence of t-butanol (5% v/v), comparing it to the case without the alcohol. The solid line in Figure 2 is a nonlinear least squares fit to equation [2] below.

If substrate inhibition is operating, it can be accounted for in the simplest manner by means of the following:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \underset{+}{\overset{S}{ES}} \xrightarrow{k_3} E + P \qquad [1]$$

$$ES \underset{k_5}{\overset{k_4}{\rightleftharpoons}} ES_2 \quad \text{(inactive)}$$

Steady state treatment of this path of reactions yields equation [2]:

Table I
Kinetic Parameters for the Hydrolysis of Phenolphthalein
β-D-Glucusoduronic Acid at 25° C pH 5.0

Volume % of t-BuOH	$k_3(E_o)$, μmoles $min^{-1}mg^{-1}$	$\frac{k_2 + k_3}{k_1}$ μm	$\frac{k_4}{k_5}$ $(\mu m)^{-1}$
Zero	$1.2 \pm 0.7 \times 10^{-2}$	8.3 ± 4	$25 \pm 10 \times 10^{-3}$
5	$3.17 \pm 0.11 \times 10^{-2}$	13.3 ± 1.5	$5.0 \pm 1.7 \times 10^{-3}$

$$v = \frac{k_3(E_o)(S)}{\frac{k_2 + k_3}{k_1} + (S) + \frac{k_4}{k_5}(S)^2} \qquad [2]$$

An analysis of equation [2], by means of taking its reciprocal and plotting, is shown in Figure 3. In the presence of t-butanol, V_{max}, which is the product $k_3(E_o)$ in equation [2], is larger than the control. But it is also seen that the dramatic substrate inhibition present in the control with no cosolvent, is considerably decreased in the presence of 5% t-butanol. A nonlinear least square fit of the data to equation [2] gives the values listed in Table I.

In proceeding from 0% t-butanol (control), to 5% t-butanol, the value of $k_3(E_o)$ increases by 2.6 fold, $(k_2 + k_3)/k_1$ increased by a factor of 1.6 and k_4/k_5 becomes smaller by a factor of 5. Although other reasonable inhibition mechanisms can surely be invoked (6), they will usually lead back to the same sort of conclusion. Namely, that substrate inhibition is an important factor, and is exhibited in the dependence of overall rate parameters on both organic cosolvent concentration and substrate concentration, at least with the most commonly used substrate.

It is known from the work of Fishman and Green (7) that glucuronyl transfer to alcohol does occur in alcohol-water mixtures. However, for t-butanol, they observed about 15% transfer. Since we are working at a lower alcohol concentration than they, we expect a correspondingly lower fraction of transfer. Moreover, transfer cannot be used to account for the stimulation of activity

by chloroform and dioxane, since these cosolvents cannot engage in such reactions.

Overall control of β-glucuronidase velocity, and perhaps its function within a cell, by imposition of solvents which in effect are different from neat water, remains an intriguing problem. Two groups, Fink (8) and Douzou et al (9), have employed organic cosolvents in cryoenzyme research in order to attempt to trap intermediates. In some of these studies, it appears that frequently the general nature of the enzyme folding is not greatly altered by rather large cosolvent concentrations (50-80%). It would be of considerable interest to extent their methods to β-glucuronidase.

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